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A Study of the Methods for Determining the Diastasic Action by Bacteria

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A STUDY OF THE METHODS FOR DETERMINING THE DIASTASIC ACTION OF BACTERIA

BY

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B. S. University of Illinois, 1917

THESIS

Submitted in Partial Fulfillment of the Requirements for the

Degree of

MASTER OF SCIENCE

IN BACTERIOLOGY

IN

THE GRADUATE SCHOOL

OF THE

UNIVERSITY OF ILLINOIS

1920

1920
F96

UNIVERSITY OF ILLINOIS

THE GRADUATE SCHOOL

June 1, 1920.

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Ruth Scovell Funk

ENTITLED A Study of Methods for Determining Diastasic Action of Bacteria.

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*Required for doctor's degree but not for master's

40393

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Introduction.

Since the determination of the diastasic action of bacteria is a point in the determination of the Group Number* of bacteria by the Committee of the Revision of Chart Identification of Bacterial Species of the Society of American Bacteriologists, and since it is used in pure culture studies, it seems advisable to study the methods which have been proposed for its determination. As is generally known, this physiological reaction has many difficulties connected with its determination. Obviously it would also be of much interest and importance to investigate the activities of an enzyme so wide spread in nature, in the animal and vegetable life. Such an investigation is beset with many difficulties. There are so many conditions which must be made constant in order that more accurate conslusions may be drawn from the premises. No claim is made that this report is exhaustive; an attempt has, however, been made into the subject to, perhaps, lay out the field and to determine the value of some of the methods which have been proposed by various bacteriologists. It is hoped that others will carry the work more nearly to completion and to determine the rest of the data needed to more accurately know the bacterial amylases.

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* The Group Number of the Society of American Bacteriologists is a convenient method for recording the salient characters of an organism. It is constructed after the Dewey decimal system for cataloguing books. A discussion of it is given be Tanner in his Bacteriology and Mycology of Foods, John Wiley and Sons., Inc. New York City. 1919

Historical.

A. Starch Decomposition by Enzymes Other Than Those of Bacterial Origin.

Starch constitutes one-half to three-fourths of the solid matter of the ordinary grains, and at least three-fourths of the mature potato. Unchanged starch occurs in distinct granules, differing in different plants. These granules are not affected by cold water, but on warming, they absorb water, swell and burst, finally forming a colloidal or gelatinous mass called "starch paste". Starch, which is insoluble and indiffusible, is rendered soluble and diffusible by the digesting action of enzymes.

The discovery of these enzymes, diastases or amylases, was made in 1814 by Kirckhoff (1814) when he observed that germinating barley grains contained a principle which, when extracted, was capable of converting starch into sugar. Although this power was then thought to be due to some property of the gluten or grain, the study of it laid the foundation for the study of enzymes. Kirckhoff was also the first to study the transformation of starch by acids.

Dubrunfaut (1823) worked with the enzyme and noted that best results were obtained at 65°C--his work was the first scientific study of diastases.

Payen and Persoz, in 1833, were the first to prepare diastase from the extracts of germinated grain. They later found it in oats, wheat, corn and rice during germination. To this material, precipitated by alcohol, they gave the name "diastase".

These men were the first to ascertain the stages of action. Musculus found that dextrin and sugar were produced simultaneously by diastasic activity. In 1871-2 Griessmeyer (1871), O'Sullivan (1872), and Brüke determined that at least two dextrins were found.

In 1874, von Gorup-Besanez found diastase in several other germinating seeds, Kosmann (1877), Baranetzky (1878) and Krauch (1879), established the presence of diastase in ordinary vegetable members of the plant, other than in the seeds.

Butkewitsch (1908) has given a noteworthy contribution on vegetable diastasic enzymes. Later investigators proved the existence of two varieties of enzymes:

1. Diastase of translocation.
2. Diastase of secretion.

The diastase of translocation is the more widespread of the two diastases. It is in vegetative organs and in germinating seeds, appearing in the barley grain earlier than the other variety of diastase, and is more active at a lower temperature than the other. This enzyme dissolves the starch grains without corrosion, and is said to have a principal part in the transporting of starch from one part of the plant to another part. It has a slow action on starch paste, but readily converts soluble starch to sugar. Kjeldahl (1879) was the first to study with exactness the influence of the acidity of the medium upon the diastase.

The diastase of secretion is closely connected with germination, corroding starch grains and disintegrating them, before

solution. Smith states that this diastase is formed by a special tissue, is excreted from this tissue into a region where it may prepare nutritive substances to be subsequently absorbed by the cells, which in the first place prepared the enzyme. It rapidly liquifies starch paste, acts best at 50°-55°C, and can withstand 70°C.

Diastase is widely distributed in the animal as well as in the plant body. In 1831, Leuchs first indicated the existence of animal diastase in the action of saliva transforming starch to sugar. In 1845, Mialhe prepared diastase from the secretion of the salivary gland by precipitation with alcohol, and named the starch transforming substance salivary diastase. In the same year Bouchardat and Sandras prepared diastase from the secretion of the pancreas. In 1863, Cohnheim prepared diastase in a purer form than had heretofore been done. In 1895, Pregl proved the existence of diastase in the intestinal juice of sheep. Bial, (1894), Rohman (1892), and Hamburger (1895), gave evidence of the existence of diastase in blood and lymph. Foster (1867), in the pericardial, pleural, and peritoneal fluids, and Cohnheim and Bechamp in fresh filtered urine.

Diastase is common among invertebrates. Boudourg (1899) found the enzyme in the pyloric tubes of certain Tellostean fishes; Fredericq (1878) in the juices from sponges; Abelous and Heim (1892) in the eggs of certain crustaceans; and Müller and Masayama (1900) in the yolk of hens' egg.

Although in the animal body the two varieties of diastase have not been indicated so clearly as with the plants, two modes of action resembling those of the translocation and secretion in plants

have been found. An example of this is where the secretion of the salivary glands and pancreas are poured out into special regions of the alimentary canal to convert starch and glycogen into diffusible forms. In the case of tissues an enzyme is produced which acts only on the glycogen in the interior of the cell, and from the latter the diffusible products are removed by a species of translocation. The diastase of blood and lymph may be compared with the translocation diastase of plants.

B. Starch Decomposition by Bacterial Enzymes.

In addition to theplant and animal diastases, starch is decomposed by the hydrolytic, extracellular, bacterial enzyme diastase. In 1882, Wortman showed that bacteria have the property of secreting certain starch dissolving ferments. Because these bacteria were able to cause a change in starch paste and starch grains as well as in soluble starch, he concluded that the action of bacteria upon starch is by a ferment which is soluble in alcohol and water. Also, that bacteria show diastasic powers when starch grains are their only available food.

In 1883 Duclaux worked on the saccharifying power of Aspergillus niger. In 1890 Fermi recorded the examination of many species of bacteria for diastase, and found that the Prior's bacillus, Koch's cholera vibrio, Bacillus ramosus, Bacillus megaterium, and a spirillum of cheese gave a positive test for diastase.

Cavazzani in 1893, while working with an organism which he identified as probably being Bacillus magdis, found that it had the ability to convert starch into glucose. In 1895, Bourquelot and Herissey demonstrated the presence of diastase in the tissue of the fungus, Polyporus sulphurous. De Bary (1858) showed the presence of the enzyme from Aethalium, a myxomycete; Morris and Wells in yeast cells; Lauder, Brunton and MacFadyen (1889), and Wortman (1882) isolated it from bacteria, when grown on starch paste. Pfeffer (1896) noted the secretion of diastase by Bacillus megatherium. In 1901, Kohnstamm showed that fungi as well as bacteria secrete a starch destroying enzyme.

In general, the action of bacterial enzymes is the same as that by digestive enzymes, etc. Starch which has the same chemical formula as cellulose is broken up into soluble sugars in the same way. The process, which is a gradual one, goes thru the cleavage products of dextrins and is then hydrolyzed to maltose. This hydrolysis may be expressed in chemical symbols as:

$$2(C_6H_{12}O_6) + N(H_2O)_n + \text{diastase} = n(C_{12}H_{22}O_{11}) + \text{diastase}.$$

As a rule, enzyme formation and bacterial growth run fairly parallel, but not always. Bacteria do not uniformly contain diastase nor is it present at all times in those which secrete it. In certain media, bacteria will grow, but do not form enzymes until, according to Jacoby (1917), certain other minerals (e. g. leucine) are added. He also states that leucine is necessary for the formation of the enzyme urease, but has no effect on its activity.

B_2. Significance in Pure Culture Study.

These diastasic enzymes are of significance in pure culture study because of the aid they give in the classification or grouping of organisms thru certain characteristic reaction, as those called for in the Group number. By this physiologic function microörganisms either are or are not classed as those producing diastasic enzymes.

As stated by the Classification Committee of the Society of American Bacteriologists, the diastasic action presents the greatest difficulties of the various physiological reactions selected for the determination of an organism.

B_3. Significance in the Industries.

Diastasic enzymes may be of importance in the manufacturing of alcohol, wines, etc. The conversion of starch into maltose by the amylase of certain molds is of importance in the manufacturing of industrial alcohols. Several species of mucor, particularly M. rouxii and M. oryzae, and one species of aspergillus, A. oryzae, have been used for this purpose. Mucor boulard No. 5, has been isolated from the mucors and bacteria on decomposing vegetable matter, especially that containing sugar and starch. In this way is formed from the starch, the sugar upon which yeast is to act. "The mucor rouxii is the characteristic mold of Chinese or Java yeast". The Japanese beer sake or rice beer is brought about by Aspergillus oryzae. "Pombe from Africa is produced by Schizosaccharomyces pombe." The production of diastase in seeds during germination is used in the process of malting and brewing. "Takadiastase, a mixture of saccharifying enzymes from Asperigillus oryzae, a fungus called koji-yeast, saccharifies starch more energetically than malt-diastase does." Bread making is another instance of economic importance in which the diastase from grains used convert some of the starch to sugar. The diastasic enzymes are of biological importance as shown somewhat by their occurrence. These enzymes as discovered by Claude Bernard, are widespread in the animal kingdom. They are in blood serum, muscle plasma, body fluids, organs, and also have been found in hens' eggs.

C. Methods Which Have Been Used for Determining Diastasic Action by Bacteria.

Various methods have been used for determining diastasic action by bacteria. Perhaps the one most universal in use now is Allen's plate method. This consists of preparing a starch agar by adding 0.2% water soluble starch to a standard agar, sterilizing in the autoclave, pouring into petrie plates, and allowing to cool. After this, a streak two inches long is made with the organism being studied, after which the plates are incubated two days at 32°C, and five days at 20°C, then flooded with a saturated solution of Iodine in 50% alcohol. A clear zone averaging more than 2 mm. in width is classed as strong, while a width of 2 mm. or less is marked as feeble, and the absence of a halo as absent.

Edson and Carpenter (1918) found that a thin starch paste containing 2% thymol did not show diastasic action.

A nutrient Starch Jelly for the Study of Diastasic Action has been prepared by adding 1 gm. of clear aseptic potato starch to 10 c.c. portions of modified Uschinsky's Solution, plugged and heated for two hours for five successive days at 85°-93°C.

Müller (1908) used a method of placing the ferment solution in increasing amounts in test tubes, adding 5 c.c. of 1% starch paste to each tube and placing immediately in ice water until all were ready. The tubes were then placed in a water bath at 40°C for 30-60 min., diluted with water, and a drop of N/10 Iodine added and shaken. The strength of the ferment is fixed as that number of c.c. of starch paste which 1 c.c. of ferment solution can transform into dextrin in the given time. (Saliva was used as the ferment).

Thatcher and Koch (1916) used a method for determing the diastasic activity of extracts in which bacteria have been grown by adding to 25 c.c. of 10% Lintner's starch solution of known acidity, N/20 alkali and 20 c.c. of enzyme solution (extract) giving the optimum acidity for enzyme activity. This was placed at 40°C for 30 min. and the enzyme activity stopped by bringing the acidity to N/20 acid, cooled to 20-22°C to precipitate proteins, an aliquot reduced with Fehling's and the amount of reduced copper determined by the Iodine method. The difference between the amount of copper reduced and that in the blank was considered due to the diastases secreted by the bacteria.

Crabill and Reed (1915) used a stock agar consisting of:

1000	c.c.	distilled water.
0.5	gm.	magnesium sulphate.
1.0	"	di-potassium hydrogen phosphate.
0.5	"	potassium chloride.
0.01	"	ferrous sulphate.
20.0	"	agar.

To this filtered agar was added 20 gm. of corn starch in suspension in a little cold water sterilized in flasks and poured with frequent shaking directly into the sterile petrie dishes. As soon as hardened, the centers of the dishes were inoculated with the test organisms, inverted, and incubated from 2 to 5 days under bell jars.

Waksman (1919) used the tube andplate method for the study of diastasic production in connection with the Studies in Metabolism of Actinomycetes, with Czapek's Solution (1 gm. K_2HPO_4, 0.5 gm. M_9SO_4, 0.5 gm. KCL, 0.01 gm. Fe SO_4, 2 gm. Na NO_3 to a liter of water).

In the tube method, 10 gm. of starch boiled in distilled water was added to a solution of the minerals, and the volume made to a liter. This solution was placed in test tubes of equal size, 10 c.c. to a tube, sterilized and inoculated. A control was compared with the inoculated incubated tubes, and the difference of heights of starch in the test tubes was taken as the diastasic action of the organism.

In the plate method, 1.2% agar was added to the liter of medium and poured into sterile petrie dishes (10-12 c.c. to a plate). These were cooled, inoculated, incubated and tested with Iodine and Potassium Iodide.

A study of the destruction of potato starch was made on sterilized potato cylinders in test tubes upon which cultures were then crushed in porcelain mortars and 50 c.c. of water added to separate the particles so that the effect of Iodine could easily be seen. The presence of unchanged starch was determined by the gradual addition of a weak solution of Iodine and Potassium Iodide.

After a sufficient amount was added to satisfy some of the other compounds which were present the starch grains turned a blue-black. The extent of the reduction of the starch by the bacterial growth is estimated on the basis of similar tests of uninoculated potato cylinders. This method was also worked on by Dr. E. F. Smith (1905).

Müller tested diastasic ferment action by keeping a mixture of starch and water (about 10%) at 55° for from 1 to 2 days, stirring, pouring into petrie dishes, closing and keeping in a hot air bath at 85-90° for from 1 to 2 hrs. The excess of water was poured off; after cooling, the inoculum was put upon the surface,

and the furrow caused by liquefaction of the paste was the evidence of enzyme activity.

Chemistry of Starch. (Biochemistry).

A. Why Starch has to be Broken Down and Why Bacteria Break It Up.

Starch in its original form cannot be used by cells because they depend upon soluble food, since they have no means of incorporating insoluble compounds. This starch, which serves in the nutrition of almost all animals and plants, is not directly assimilable. All living cells secrete enzymes, and these agents dissolve or hydrolyze the insoluble starch to maltose by means of the enzyme diastase. This extracellular, hydrolysis process yields very little energy, but is a preliminary process to the getting of energy. The product, maltose, is further acted upon by intracellular enzymes or by oxidation, and much energy is evolved. The excess of energy in this process over that used in the hydrolysis is the energy available for the cell in building up new tissue or cell protoplasm.

This breaking down of starch by enzymes is then a preparatory process to the securing of energy, as a result of which process, digestion or fermentation takes place, and energy available for cell use is given off; also the substances formed by the diastases are assimilable and suitable for the formation of new tissue by the cells. (This preparatory process, however, has no direct relation to the intracellular food digestion or fermentation). Starch then must be broken down to become utilizable by cells, and bacteria break it up to obtain energy.

B. Chemistry of Starch Decomposition by Enzymes.

The size and chemistry of the starch molecule has been the subject of much chemical research since about 1836 when Payen announced the chemical composition to be $(C_6H_{10}O_5\cdot)_n$ This imperical formula is generally accepted at the present time, but it is very hard to prepare starch entirely free from phosphoric acid. It is possible that this mineral (acid) is in union with the starch molecule. Certain facts about starch indicate a high degree of complexity which probably approaches that of the proteins. Among these facts are its highly colloidal nature, the ease with which it can be removed from its solutions by forcing through earthenware, and the small influence which it exerts upon the freezing point of its solvent. "The $(C_6H_{10}O_5)_n$ structure indicates that starch is an anhydride condensation product of glucose or maltose."

"The chemical process of hydrolysis of starch presumably takes place in several phases, in which the dextrins formed as intermediate products are broken down. Amylase may consequently be composed of several enzymes."

The close relationship between enzymes and their substrate indicate that enzymes enter into a chemical combination with the molecules on which they work. Enzymes also enter into combination with dissociation products. Investigations on diastase indicate that enzymes are made up of two parts, a combination of a colloid with an active principle. The colloid is generally related chemically with the substance on which the enzyme acts (substrate). So long as the enzyme is combined with its carrier it remains inactive, but is broken away by slight changes in reaction.

Tebb (1894), Brown, Morris Röhmann (1894), Hamburger (1895), and Beijerinch (1895) detected in the mixtures of starch saccharifying enzymes, one that transforms starch to maltose, and another that breaks down maltose to glucose. Wijsman (1890) assumed that the reactions, starch to dextrin and dextrin to maltose, were brought about by separate enzymes, and more recently Ascoli and Bonfanti (1904) considered several enzymes as bringing about the change. Although diastase is usually considered as one enzyme, it might more properly be considered as consisting of two classes of enzymes, one which hydrolyzes the starch to soluble starch, dextrins and maltose (amylase), and the other which hydrolyzes the maltose into dextrose (maltose). Brown and Morris (1893) stated that at least two enzymes showing different biological relations, diastase of translocation, and diastase of secretion, exist. Later, Maquenne explained saccharification from a chemical point of view. In this explanation it must be kept in mind that starch consists of 80-85% amylose and 15-20% amylopectin.

Amylose is rapidly dissolved when starch is treated with boiling water for a few minutes, gives a blue color with Iodine, and forms no starch paste. It is readily attacked and dissolved by amylase whereas amylopectin is very slowly acted on by this enzyme. "In the saccharification of pure amylose, maltose alone is formed". Amylose is the last of a series of condensation products increasingly soluble in water, the lower members of which are largely contained in soluble starch. Samec (1916) states that the term "soluble starch" is indefinite and does not adequately define the substance. He also states that in some cases the size of the molecule was reduced, but in others the molecule was

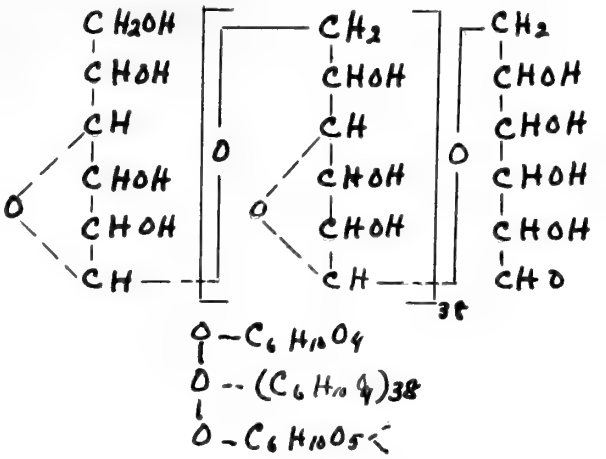
not broken down, and that most forms did not contain phosphorous.

Amylopectin is a gelatinous substance, purplish red with Iodine, insoluble in water and alkalies, and on hydrolysis, gives dextrin as well as maltose. It is probable that the enzyme amylopectinase and not amylase saccharifies amylopectin (starch paste) with ease. The "gelatinazation" of starch caused by hot water probably results from the simultaneous solution of amylose and the swelling of amylopectin.

Of these two substances resulting from the breaking down of starch, amylopectin contains all of the phosphorous from the starch molecule, while the amylose contains only a slight trace. The phosphorous pentoxide content of starch according to Samec decreases with the viscosity of starch, but is not broken down by diastase. The diastases of plants and animals must contain both diastatic enzymes. Starch decomposition by enzymes is a hydrolytic process and may be represented by the equation: $2\ (C_6H_{10}O_5)_n + (NH_2O) = NC_{12}H_{22}O_{11}$. This maltose formed is acted upon by the enzyme maltose giving dextrose. $C_{12}H_{22}O_{11} + H_2O$ + maltose = $2\ C_6H_{12}O_6$ + maltose. Then alcoholase, a zymase, may act upon the dextrose giving alcohol and carbon dioxide. Experiments by different authors regarding the hydrolysis curves do not agree. A differentiation may be made, in the action of diastasic enzymes upon starch, between the amylolytic (amyloclastic, liquefying) and the saccharogenic power.

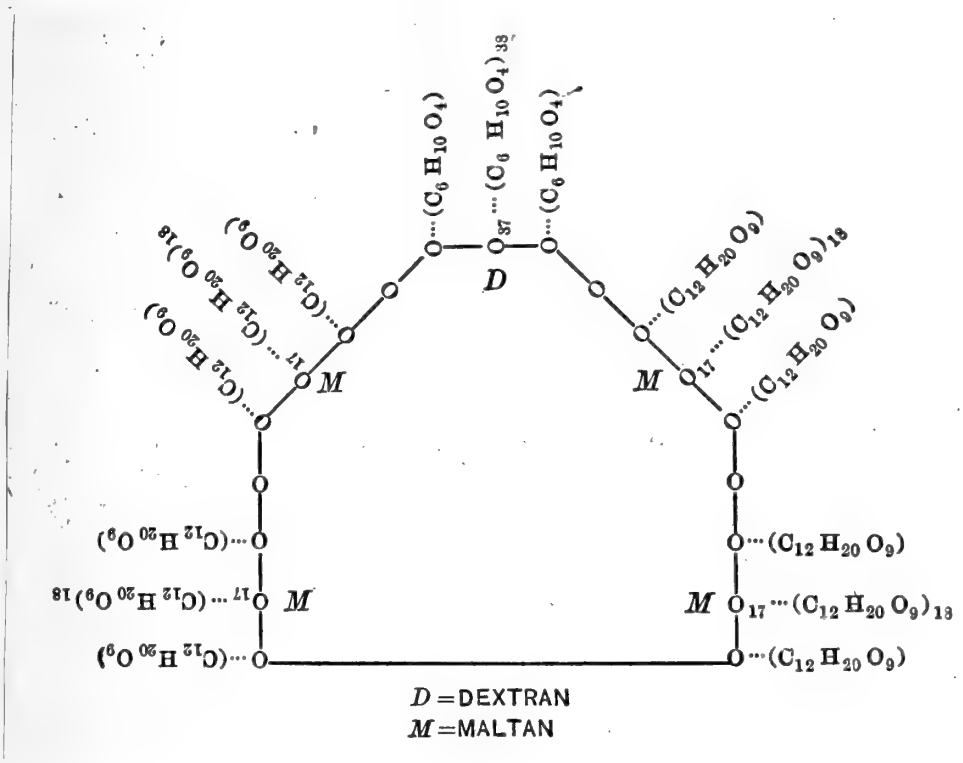
A history of the study of the starch molecule shows variation as to the size of the molecule. In 1860 Musculus noted that it was difficult to completely hydrolyze starch to maltose by means

of malt and later (1878) decided that starch must be a polysaccharide with a molecular size of $(C_{12}H_{20}O_6)$. Herzfeld (1879) pointed out the fact that starch hydrolysis progressed through a series of dextrins of diminishing complexity i. 4. starch = soluble starch = erythrodextrin = achroodextrin = malto dextrin = maltase. To malto dextrin, he gave the formula $C_{18}H_{36}O_{16}$. Brown and Heron (1879) concluded that the starch molecule must be at least five times the size of the residual dextrin and proposed $(C_{12}H_{20}O_{10})_{10}$ as the formula for starch, and $(C_{12}H_{20}O_{10})_2$ as that for the simplest dextrin molecule. In 1855 Brown and Morris reported that dextrin was always present as one of the hydrolytic products of starch. To starch they gave the formula $5(C_{12}H_{20}O_{10})_3$. Upon hydrolysis an amylin group $(C_{12}H_{20}O_{10})_3$ was thought to split off as maltodextrin and this in turn to maltose, leaving the four other amylin groups as a more complex dextrin. Lintner and Duell (1893) claimed that in the hydrolysis of starch the complex starch molecule was split first into amylodextrin (soluble starch) $(C_{12}H_{20}O_{10})_{54}$ then into three molecules of erythrodextrin $(C_{12}H_{20}O_{18}H_{20}$ which in turn split into three molecules of achroodextrin $(C_{12}H_{20}O_{10})_6H_{20}$, and then into isomaltose, the isomaltose changing to maltose. In 1899 Brown and Millar discovered a reducing dextrin to which they gave the formula $39(C_6H_{10}O_5) + (C_6H_{12}O_6)$ or $40(C_6H_{10}O_5) + H_2O$. "It might be made up of the condensation of forty glucose molecules with the elimination of 39 molecules of water, in accordance with the appended structural formulas".



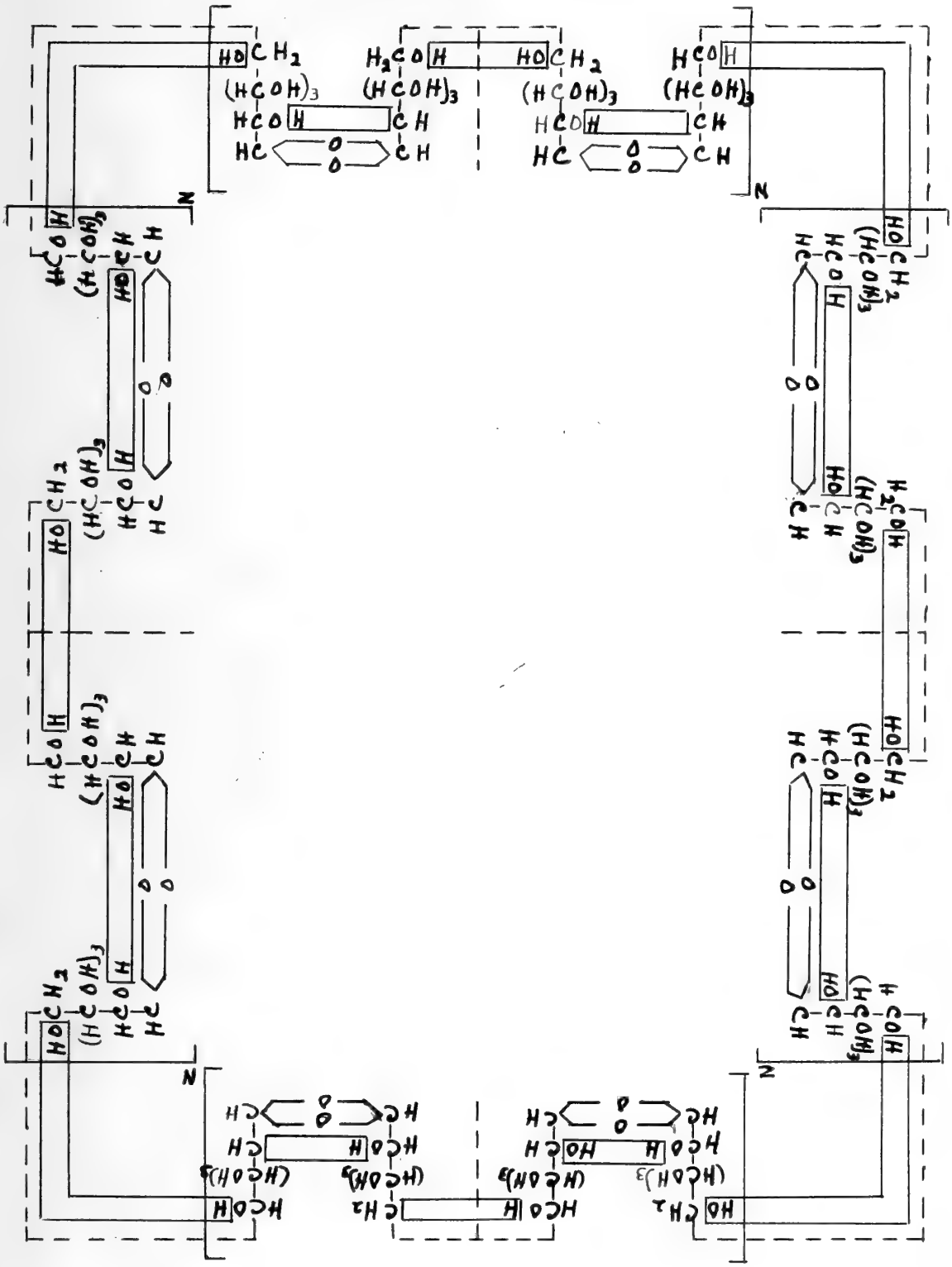
(< Signifies a free aldehyde group.)

These investigators state that the simplest way to express the constitutional form is to consider it made up of the eighty residues of maltan and forty dextran groups linked in a ring form by means of oxygen atoms. According to this the starch molecule may be represented by their proposed configuration.



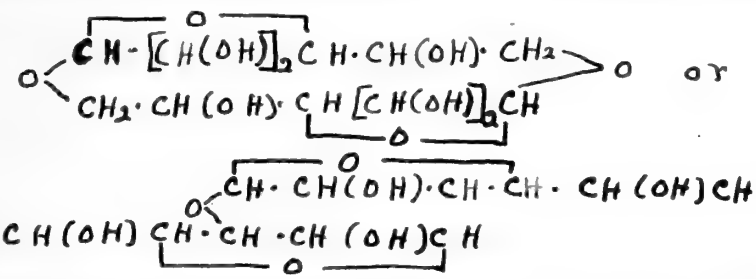
Brown and Miller's Proposed Configuration of the Starch Molecule.

Ling (1910) attacked this structure and regarded the starch molecule as $(C_6H_{10}O_5)n - (n-1\ H_2O)$ inasmuch as he considered carbohydrates to be derived from monoses by a series of condensations with the elimination of water. Johnson (1898) as a result of hypothesis proposed a formula based upon the fact that starch must be a multiple of $(C_6H_{10}O_5)_4$, these groups being arranged as follows.



Synkiewski (1902) studied the hydrolysis of starch by malt infusions. His hypothesis rests entirely on two dextrins: protodextrin I, formed by hydrolyzing starch at ordinary temperature, and protodextrin II, formed by the hydrolysis of starch at 78°C. He gives $(C_6H_{10}O_5)_{36}$ as the configuration of the starch molecule. Other structures have been proposed by Hale (1902) $(C_6H_{10}O_5)_{18}$; Kladiaschwili (1904) $(C_7H_{10}O_6)_6$; and Skraup (1905) $(C_6H_{10}O_5)_{46}$ to $(C_6H_{10}O_5)_{50}$.

The theory of the great molecular complexity of starch has been more firmly established by the work of Pringsheim (1912-15) who by fermentation and hydrolysis of starch under different conditions obtained several dextrins which were tetra- and hexapolymers of the simple dextrin molecule. From his experimental results he concluded that these dextrins or "amylases" have a ring structure such as



and that they indicate that starch itself, and the non-reducing dextrins which are not fermented by heast contain the ring which can be opened only be special ferments.

The Diastasic Enzymes.

Diastase or amylase, one of the most common eyzmes in nature, is present in all green plants, especially in germinating seeds, in shoots and leaves and other portions where starch is stored. In very young plants the germ is surrounded by storage substances used for food in growth. Among these substances is starch which is digested by enzymes, secreted by the growing germ, and which act outside ofthe protoplasm. It is said that the enzyme diastase hydrolyzes starch from grains more readily than starch from the legumes.

This type of enzyme is also found in the secretions of animals as in saliva, pancreatic juice, and in the liver. Here these enzymes play an important rôle in digestion. Muscles also are rich in this enzyme which attacks glycogen.

Amylases or diastases include all those enzymes which break down starch and glycogen forming maltose. Euler and Pope (1912) state that very little that is definite can be asserted with regard to the individuality of the diastases (amylases).

Of these enzymes, two forms are distinguished "according to the type of corrosion of the starch grain": diastase of translocation which occurs in plants, especially in the chlorenclyme of leaves and which corrodes the grains evenly: and diastase of secretion which occurs especially in the seeds and corrodes the grains unevenly. When dialyzed in spring water, the dissolved diastases are separated into two principal groups, the saccharifying diastases which diffuse through a membrane, and the liquifying ones. which remain.

In the simple organisms, as bacteria, as well as in the higher plants, the secretion of diastase has a direct bearing upon the question of nutrition of the organism. Brown and Morris (1890) state that starvation is a stimulus to this secretion; Wortmann (1882) and Lauder, Brunton and MacFadyen (1889) that diastases are secreted when starch is the only food. With animals it is said to be largely dependent upon the nervous impulses.

"Amylase can assume at the same temperature two distinct forms: a soluble form immediately saccharified and colored by Iodine, and a solid form which resists malt and gives no reaction with Iodine. The latter is perhaps a polymeric form of the first."

As a result of the study by Koch (1916), several conclusions were drawn:

That diastasic eyzmes are secreted by bacteria quantitatively.

That there is a considerable variation in their activity on different media.

That the enzyme activity is variable from day to day.

That there is no direct correlation between hydrolytic enzyme secretion and protein decomposition by bacteria, and

That enzyme activity of different species varies greatly.

The action of diastase is in many respects similar to that produced by acids acting on carbohydrates. Various diastases differ considerably among themselves as to nature and sensitiveness towards reagents.

The greater or less sensitiveness of diastases to the action of antiseptics and to those substances paralyzing the vital activity can be utilized to exclude the activity of microörganisms during diastasic action. Cellular activity of a cell may be de-

stroyed, yet it may secrete diastase. Different diastases may be characterized by their optimum temperature. Payen found a general property of enzymes and a general means of isolation in their property of being precipitated from solution by alcohol. (Some diastases dissolve easily while others require a larger amount of water).

Chrzosoz (1908) showed that both the saccharifying and liquifying action of the diastases are often parallel.

"Work with different diastases and different starches under varying conditions tend to show that soluble starches are not all attacked to the same degree by the same diastases at the same temperature."

"Lang (1911) has shown that different starches of different origins react differently to pancreatic diastase.

Ehrich (1916) states that raw and gelatinized potato starch is more readily saccharified than barley starch, and the latter more easily than gelatinized malt starch.

In work on hydrolysis curves, it has been found that the initial speed of conversion for a constant amount of enzyme was the same for different concentrations of starch, and that the reaction diminished the more rapidly, the smaller the initial concentration of the starch.

It has been found that certain acids and salts have an influence upon the activity of diastases. Sherman and Thomas (1915) found that "weak acids (acetic, propionic, phospheric) strong acids (hydrochloric, nitric, sulphuric) and acid phosphates

(sodium and potassium) produced optimum activation of malt diastase in concentrations corresponding to the same actual acidity, P_H = 4.2 determined electrometrically.

According to Lintner and others, the action of malt diastase is accelerated only by very small quantities of weak acids. A concentration of acid as low as 0.001% of Hydrochloric causes retardation. With pancreatic diastase, rather high concentrations of Hydrogen ions (about 0.001 N) are necessary for the activity to reach its maximum.

Pancreatic, malt, and aspergillus oryzae amylases react favorably in the presence of salt and phosphate. With these, considerable dextrin remained, indicating that dextrin, maltose, and glucose may not be the only products of the action of amylases on soluble starch. The amylase of aspergillus oryzae exerts its maximum activity both amyloclastic and saccharogenic, in a very slightly acid medium. Acid phosphates accelerate and alkaline phosphates retard the action. Neutral electrolytes (sodium chloride, potassium chloride) have no measurable effect on the saccharogenic action, but increase the amyloclastic action of commercial taka-diastase. "When saccharification and fermentation are both conducted simultaneously, the addition of small quantities of calcium salts help to prevent any refractory starch particles from escaping dissolution, while its retarding influence on saccharification is of no serious importance."

Sherman and Walker (1917) found that the action of malt amylase was increased when the electrolyte (hydrochloric, phosphoric acid, potassium, di-hydrogen ion, $C_H 10$ - 4.4), also that when the

concentration of the enzyme was increased, the effect of the electrolyte was decreased.

Sherman, Thomas and Baldwin (1919) confirmed earlier findings on the optimum hydrochloric ion concentration for malt amylases by using the Clark cell and rocking electrode.

They found that pancreatic amylase is active between P_{H4} & 10 with an optimum at 7; malt amylase is active between P_H2.5 & 9 with an optimum at 4.4 - 4.5; and amylases of aspergillus oryzae between P_H2.6 - 8.0 with an optimum at about 4.8. Also that the influence of the concentration of the electrolyte, as distinguished from the concentration of the hydrogen ion alone is greatest in the case of pancreatic and least in that of aspergillus oryzae amylase.

Adler (1916) found that malt diastase is active at 20°C at a hydrogen ion concentration of P_H4.9 as determined by the reduction of Fehling's solution and color reduction of Iodine solution.

Contrary to the cholrides, nitrates, sulphates, and phosphates of Sodium and Potassium, the bromides show an inhibitory effect on the activity of malt amylase when present in small amounts and an activating effect when their concentration is increased.

Potassium permanganate, hydrogen peroxide and iodine in very small concentrations inhibit the action of diastase. Ordinary formaldehyde accelerates the action slightly, but this acceleration is due to the formic acid it contains. If neutralized, it has a slightly inhibitory effect.

In the action of malt amylase upon soluble starch, there is no "region of linear relationship" in which the yield of reducing sugar is directly proportional to the time.

EXPERIMENTAL.

This investigation had its origin in an attempt to secure a better method for determining the diastasic action of microörganisms, than those which are now being used by microbiologists, and also to better understand the conditions under which those methods now in use yield most reliable results. Such a study, on account of the multiplicity of governing conditions, soon becomes rather intricate and it is not easy to select combinations which yield the best results. In order to preserve coördination among the experiments carried out and to keep the data collected in easily accessible form the work has been divided into separate parts as follows:

A. Effect of different amounts of starch on the action of diastasic enzymes on the starch agar plates.

B. Effect of concentrated agar solutions on the action of the diastasic enzymes on the starch agar plate.

C. Effect of the length of incubation period on the action of diastasic enzymes on the starch agar plate.

D. Effect of different starches in the action of diastasic enzymes on the starch agar plate.

E. Study of diastasic action in synthetic media.

F. Study of different methods used in determining diastasic action.

G. Study of varying the amount of peptone on the diastasic action of bacteria, using the dot and streak inoculation.

A. In order to determine the effect of different amounts of starch on the action of diastasic enzymes in starch agar, different percentages of potato starch were added to agar according to

the following table:

250 gm. plain agar	.50 gms. soluble starch	.2%
"	1.25 "	.5%
"	2.50 "	1.0%
"	3.75 "	1.5%
"	5.00 "	2.0%
"	7.50 "	3.0%
"	10.00 "	4.0%

The starch agar was placed into tubes, 10 c.c. to a tube, sterilized, poured into sterile Petrie dishes, allowed to harden, cooled and streaked with the test organisms, B. mesentericus, B. vulgatus, B. cereus, making one streak across the center of the plate. The plates were then inverted, incubated at 37°C for 3 to 4 days, flooded with Iodine, and the diastasic action determined in millimeters by the width of zone of liquefaction. Before adding the Iodine, the width of growth of the organism was measured in millimeters, and after flooding, the action was determined by the same scale. The averages of action are given on Table Ia.

After having determined the diastasic action of the test organisms on percentages of starch from .2 to 4.0, the same procedure was used to determine the action on smaller percentages of starch as follows: 0.1, .05, .025,.020, .015, .010 per cents the results of which are given in Table Ib.

With the lower percentages (.025, .20, .015, and .010 per cents) the amount of starch present was so small that the diastasic action with Iodine was hard to distinguish. A gradual deepening in color resulted with the increasing percentages of starch. This shows that there was not enough starch present in the four lowest percentages to give satisfactory results. With all of the percentages used, it was found that better results were obtained by allowing the agar plates to cool and harden for some time, perhaps

Table Ia. Diastasic Action with 0.2% - 4.0% Starch Inclusive and 1 1/2% Agar.

Organism.	Percentages of Starch.						
	4	3	2	1.5	1.0	0.5	0.2
B. mesentericus	*41	45	50	42.5	61.5	57.5	58
B. cereus	25	40	51		42.5	2.75	Entire
B. vulgatus		160	31	40		47.5	39.5

*Figures indicate width of zone of diastasic action in millimeters.

Table Ib. Diastasic Action with .01%-1.0% Starch Inclusive and 1 1/2% Agar.

Organism.	Percentages of Starch.					
	0.1	.05	.025	.02	.015	.01
B. mesentericus	*70	90	60	65	62.5	55
B. cereus	3.3	37	90	40	42.5	42.5
B. vulgatus	50	42.5	25	20	45	45

*Figures indicate width of zone of diastasic action in millimeters.

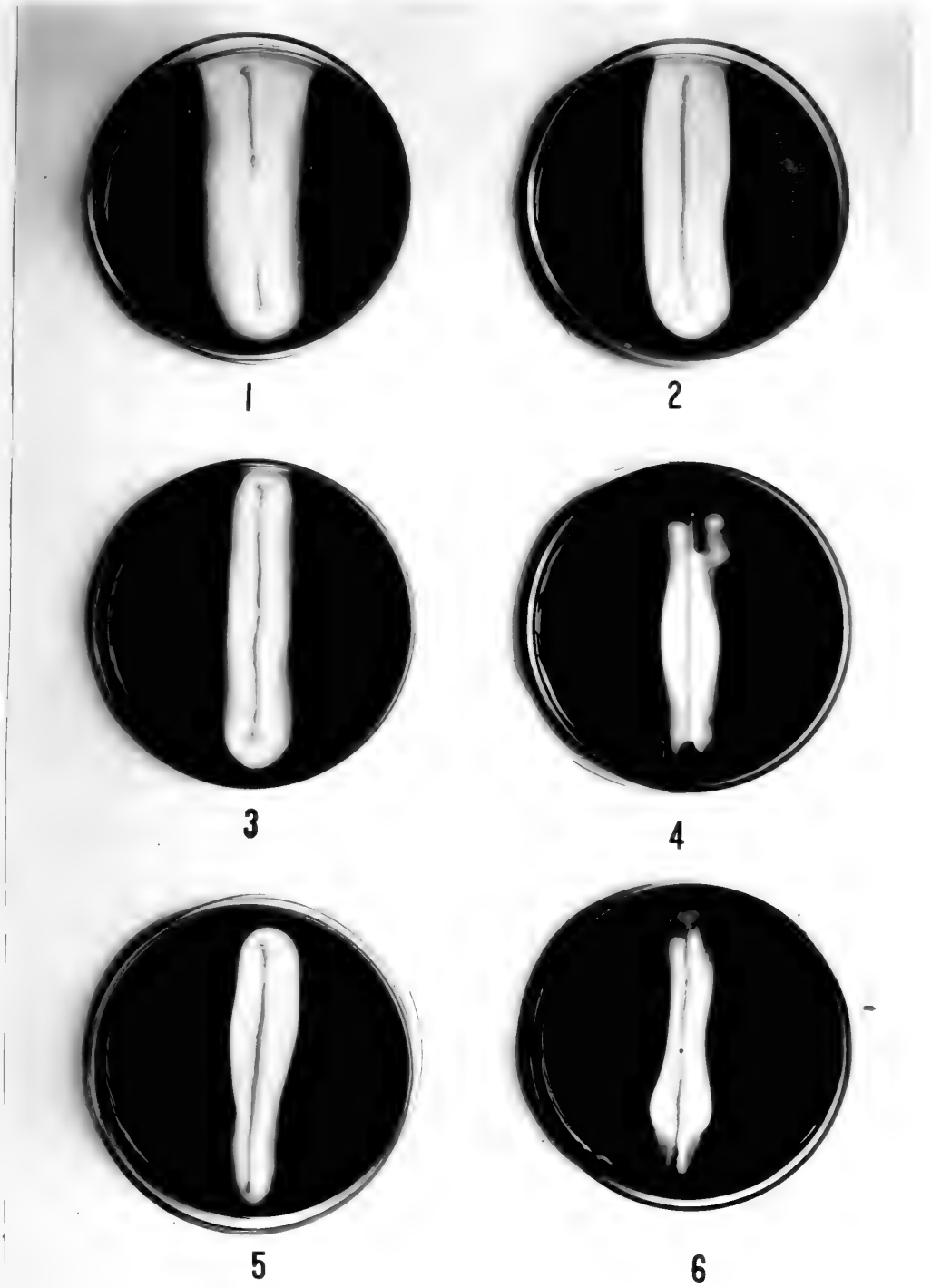


Fig. 1. Showing the Effect of Different Amounts of Starch on the Diastasic Action of B. vulgatus on 1 1/2% Agar After 4 Days Incubation at 37°C.

1 - 0.1%
2 - 1.0%
3 - 1.5%
4 - 2.0%
5 - 3.0%
6 - 4.0%

over night, before streaking. When this was done the streaking was more uniform, since the starch agar was firmer and the wire did not break through as it tended to do when the plates were freshly poured. It was also found that spreaders, when present, had usually developed after twenty-four hours. The work so far, showed that the best results were obtained between 2.0% and .5% of starch. Within this range, there was probably the proper concentration of starch to be attacked by the enzymes.

B. The next step was to determine whether a heavier agar might not show a sharper diastasic action since the enzymes would not diffuse so rapidly and their action be limited to a small distance from the line of inoculation. To prove this, 0.5, 1.0, 1.5 and 2.0 per cents of potato starch were added to two and one-half per cent agar.

The same technique was used except that in working with the heavier agar it was found to be necessary to pour the agar while quite hot, or otherwise it would harden before covering the surface of the Petrie dish. When cooled to the temperature at which 1 1/2% agar could be cooled before pouring, the 2 1/2% agar became too thick to give satisfactory results. By pouring the agar hot, leaving the covers of the dishes tipped for a short time (perhaps 5 minutes) then placing the dishes at 37°C with covers tipped for one-half hour, good results were obtained, although in some cases contamination resulted. The firmer agar allowed better inoculations to be made.

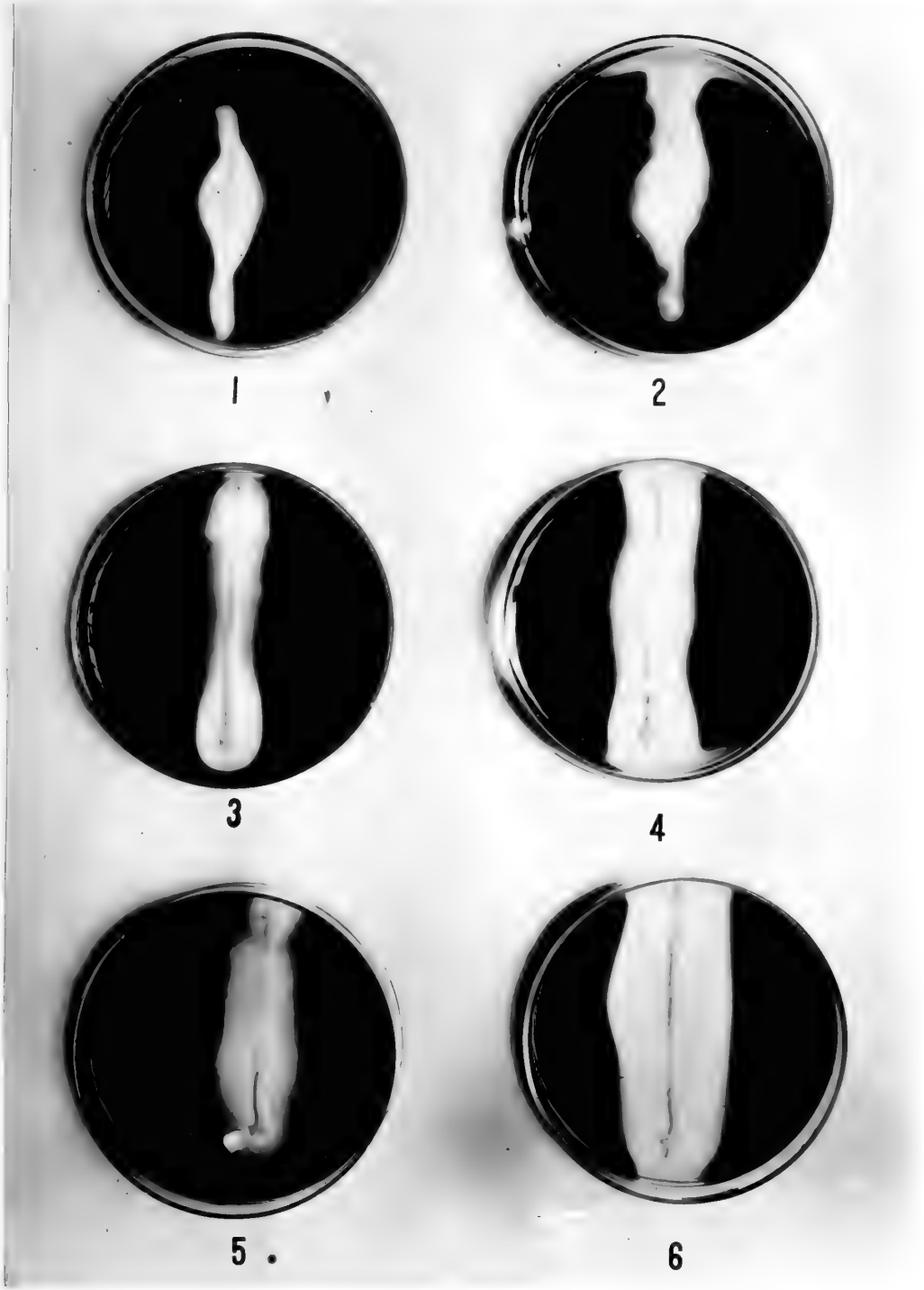


Fig. 2. Showing the Effect of Length of Incubation Period (37°C.) on Diastasic on 1 1/2% Agar and 2% Potato Starch with B. vulgatus.

1- One day.
2- Two days.
3- Three days.
4- Four days.
5- Five days.
6- Six days.

After thus disposing of the excess moisture, the plates were streaked with the test organisms, incubated at 37°C for six days and tested, the results of which are shown in Table II.

When these plates were observed the first day, no spreaders had developed as they had in 1 1/2% agar. By the third day, however, a few had developed, but not as many aswith the smaller percentage of agar. On testing with Iodine, a clear cut line of diastasic action resulted. In almost all of these plates a purple color was present at the edge of the clear zone, showing that the more concentrated agar solution had prevented the complete action of the diastasic enzymes. It is probable that the heavier agar prevented the diffusion of the enzymes making it act within narrower limits than with the one and one-half per cent agar.

Table II. Action in Millimeters on 2 1/2% Agar with percentages of Starch from 0.5% to 2% inclusive.

Organisms.	Percentages of Starch.							
	2.0		1.5		1.0		0.5	
	*Gr.	*A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	48.5	52	52	54	56.5	57	39	46.5
B. cereus	22.5	25	21.5	27	18.5	22.5	17	27
B. vulgatus	12	26	12	26	9	29	9	32.5

*Gr. = Growth in millimeters.

*A = The width of clear zone in millimeters.

C. The value of any test for determing the cultural or marphological characteristics of an organism is somewhat determined by the conditions under which the test has to be used. Usually, it is desired that a satisfactory test return results in the shortest time with the greatest accuracy. The time element is an important one in this study.

In order to determine this, 1.0 and 2.0 per cent starch in 1 1/2% agar was used with the test organisms and five series of duplicate plates tested each day for five days. B. arborescens and B. subtilis were here added to the test organisms. The averages of the growths and diastasic action are given in Table III..

On the first day's observation no spreaders or irregular growths had occurred, and the line of diastasic action was clear cut. On the second day's observation some irregular growths had taken place. Of these two observations, the 1.0% starch seemed to give the better results, i.e. the line of diastasic action seemed clearer than in the 2.0% starch. During the last three days more irregular growths resulted, and on the fifth day the 2.0% gave the better result. In all the observations there were dark specks on the clear background for about five millimeters between the clear space and the blue undigested starch. On the later observations this was more so with the 1.0% than with the 2.0% starch, but on the 1st and 2nd days the 1.0% starch gave the best results. The amount of starch to be used then seems to depend on the period of incubation used. A low concentration with a long incubation period would probably show complete destruction.

Table III. Growth and Diastasic Action on 1 1/2% Agar from 1 to 5 days inclusive with 1% and 2% potato starch.

Days	Percentages of Starch.											
	B. mesentericus				B. cereus				B. vulgatus			
	1%		2%		1%		2%		1%		2%	
	*G	*A	G	A	G	A	C	A	G	A	G	A
1.	14.5	21	12	18	5.0	11	9	10	1	12	1	10
2.	23	26	22	25	8.5	15	15	17	2	16	3	12
3.	35	38	30	36	13	22	22	25	10	24		20
4.	48.5	55	42.0	46	23	24	17	28	12	28	12	23
5.	48.5	51	46.0	50	25	36	29	36	14	31	14	24

*G = Growth in millimeters.

*A = Figures indicate width of clear zone in millimeters.

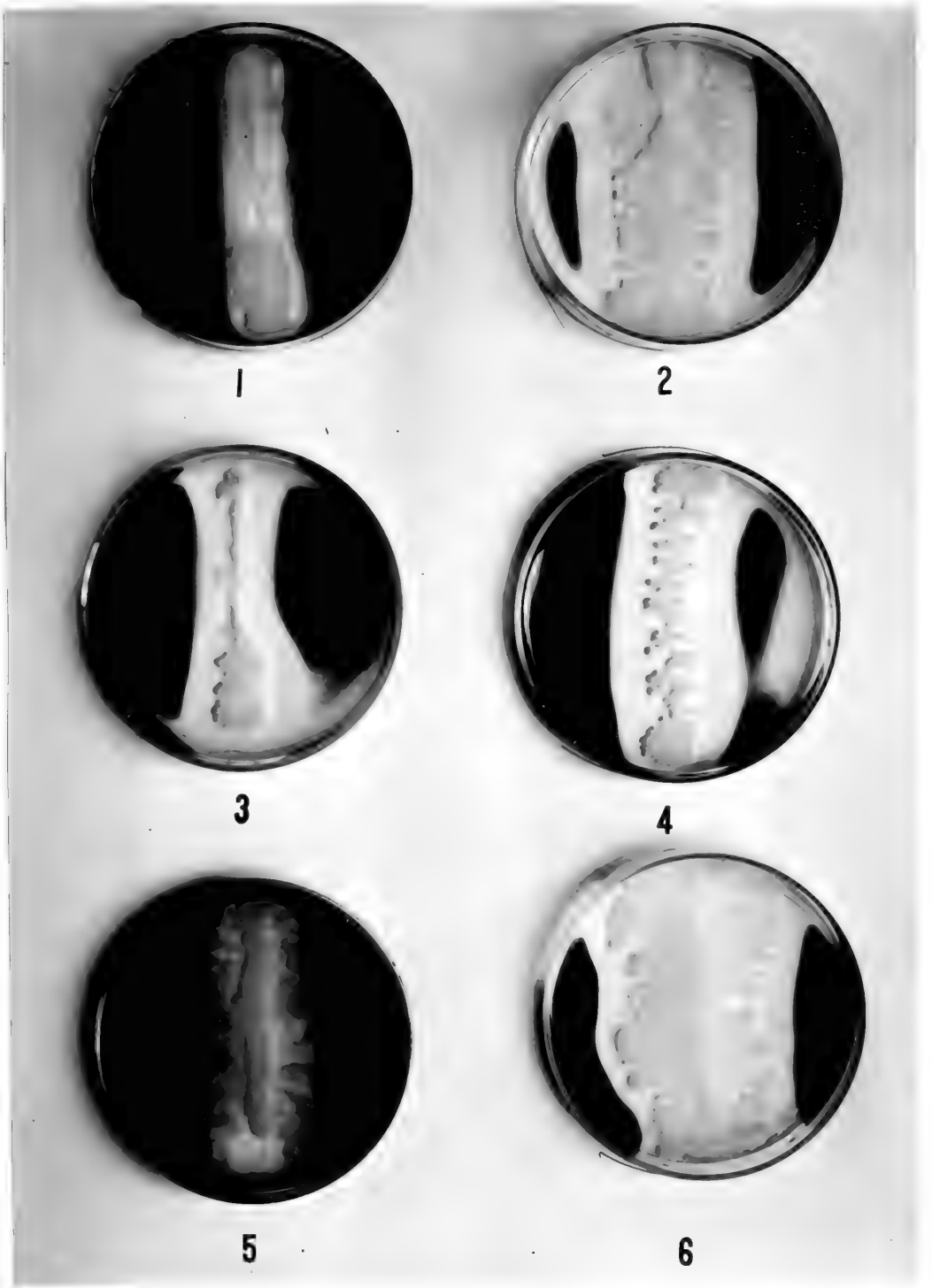


Fig. 3. Showing the Effect of Different Percentages of Agar on the Diastasic Action of Starch After Five Days Incubation at 37°C on 1 1/2% and on 2 1/2% Agar.

2 1/2% Agar.	1 1/2% Agar.
1 - B. subtilis.	1 - B. subtilis.
2 - B. vulgatus.	2 - B. vulgatus.
3 - B. mesentericus.	3 - B. mesentericus.

D. It now seemed to be important to determine whether the kind of starch had any influence on its availability for bacteria. The same technique as previous experiments was used. Plates of 2.0% agar with pea, wheat, corn, oat, arrowroot, and rice starch were prepared and observations made after one and five days.

In preparing these plates equal amounts of cold water were added to the starches to make a thin paste; this emulsion was poured slowly into the hot melted agar, stirring constantly, brought to a boil, tubed, sterilized, plated, cooled and streaked as in the previous experiment.

The appearance of these starch agar plates was different from the previous plates. With these starches, a 2 per cent starch agar plate was more opaque than the agar containing 1 1/2 per cent of starch, the zone of action could also be seen more clearly before Iodine was added. It seemed to be more transparent, or granular than the rest of the agar. This was true with the six different starches used.

As a result of the observations made after one day's incubation, the results of which are given in Table IVa, it seems probable that the starches were not soluble ones.

On the oat starch the diastasic action with all of the organisms had probably not proceeded farther than between the soluble and erythrodextrin stage, as shown by the purple color. The appearance of purple specks on the background on all of the plates used for the one day observations, except where complete diastasic action had in part taken place, was further evidence of insoluble starch particles.

Table IVa. Growth and Diastasic action after 1 and 2 days incubation at 37°C on 2 1/2% Agar with various starches.

Organisms.	One day.												Twadays.	
	Oat		Arrow-root		Corn		Pea		Wheat		Rice		Corn	
	*G	*A	G	A	G	A	G	A	G	A	G	A	G	A
B. mesentericus	4	10	6	15	3	11	4	9	6	10	5	12	10	22
B. vulgatus	2	6	4	12	3	5	2	-	3	7	2	4	5	12
B. cereus	1	6	1	10	0.5	5	1	5	1	6	2	6	4	14
B. arborescens	5	10	25	30	5	10	8	10	9	10	4	9	11	17
B. subtilis	1	8	3	10	2	8	2	8	3	9	3	10	4	18

*G = Growth in millimeters.

*A = Figures indicate width of clear zone in millimeters.

The diastasic action with the wheat starch seemed to have gone a little farther than with the oat starch, the erythrodextrin stage being visible in part of the plates.

With the corn starch the action was a little stronger, but not as strong as with the arrowroot. The zone of action was regular but not complete.

The greenish blue color with Iodine was more decided with the pea starch than with the arrowroot. With the exception of B.cereus where no action had taken place at all, the action was similar to that of arrowroot. With this starch also, dark specks were visible in the background.

With the arrowroot starch the action had proceeded in part to the achro-dextrin or maltose stage, but the whole zone of diastasic action was not clear, the dark specks again being visible in the purple zone. A greenish blue color instead of the ordinary blue with Iodine was present with this starch.

The weakest action of all was obtained with the rice starch; larger and greater numbers of dark specks were present in the zone of action than with the others, B. vulgatus being the only one with which the action had proceeded to the clear stage, the others all had a purplish tinge. This might indicate that the different starches had different resistivities to enzymic action.

According to the action, in general, from weakest to strongest, on the one day observation the starches would be arranged as follows: rice, oat, wheat, corn, pea, and arrowroot. Soluble potato starch gave the strongest action.

A two-day observation was made on corn starch to see how the result compared with the one-day. The action had in part gone to the colorless stage as shown in Table IVa, but the intermediate cleavage stages were also present. This shows that with these starches a different time element would have to be considered than for potato starch as worked out in the first experiments. For this reason similar plates were incubated at 37°C for seven days and tested, with results as given in Table IVb.

After seven days incubation at 37°C the zone of action with each organism was much more extended than after one day incubation, but with no organism had the action gone to completion. In general the action had gone farther, but the different stages of diastasic action were visible. There were specks also in the background.

With rice, complete action had taken place on part of the zone, but here as with the one-day observation larger and more particles of undigested starch were present.

In the oat starch the action had proceeded farther with the result that B. mesentericus, B. vulgatus, and B. subtilis gave a zone of clear action, first pink, then purplish; but with B.cereus and B. arborescens although four times in width, the color change was still in the light purple stage. The action with the wheat was similar to that of the oat starch.

With corn the zone of decomposition as with the other starches, was of greater width, but with only two organisms, B. vulgatus and B. subtilis,had a clear zone appeared and that only on a portion of the field.

Table IVb. Growth and Diastasic Action after seven days incubation at 37°C on 2 1/2% Agar with different starches.

Organisms.	Starches.											
	Oat		Arrow-root		Corn		Pea		Wheat		Rice	
	*G	*A	G	A	G	A	G	A	G	A	G	A
B. mesentericus	25	42	11	22	19	33	25	26	35	44	28	41
B. vilgatus	10	23	13	25	10	23	11	12	9	20	23	27
B. cereus	2	26	3	20	3	32	2	11	15	61	10	29
B. arborescens	37	42	48	49	44	49	56	57	78	79	15	30
B. subtilis	14	30	10	38	14	39	11	38	13	37	36	44

*G = Growth in millimeters.

*A = Figures indicate width of clear zone in millimeters.

Again with pea starch the same two organisms gave a clear zone on a portion of the field. None of the organisms with the arrowroot starch had proceeded to the achrodextrin stage.

This observation shows that even with seven days incubation at 37°C these different starches do not give satisfactory results as a test for the action of diastasic enzymes. The starches used evidently were not soluble ones. With the soluble potato starch prepared by Lintner's method, it is probable that the cells are ruptured and their contents more readily available for the action of the enzymes.

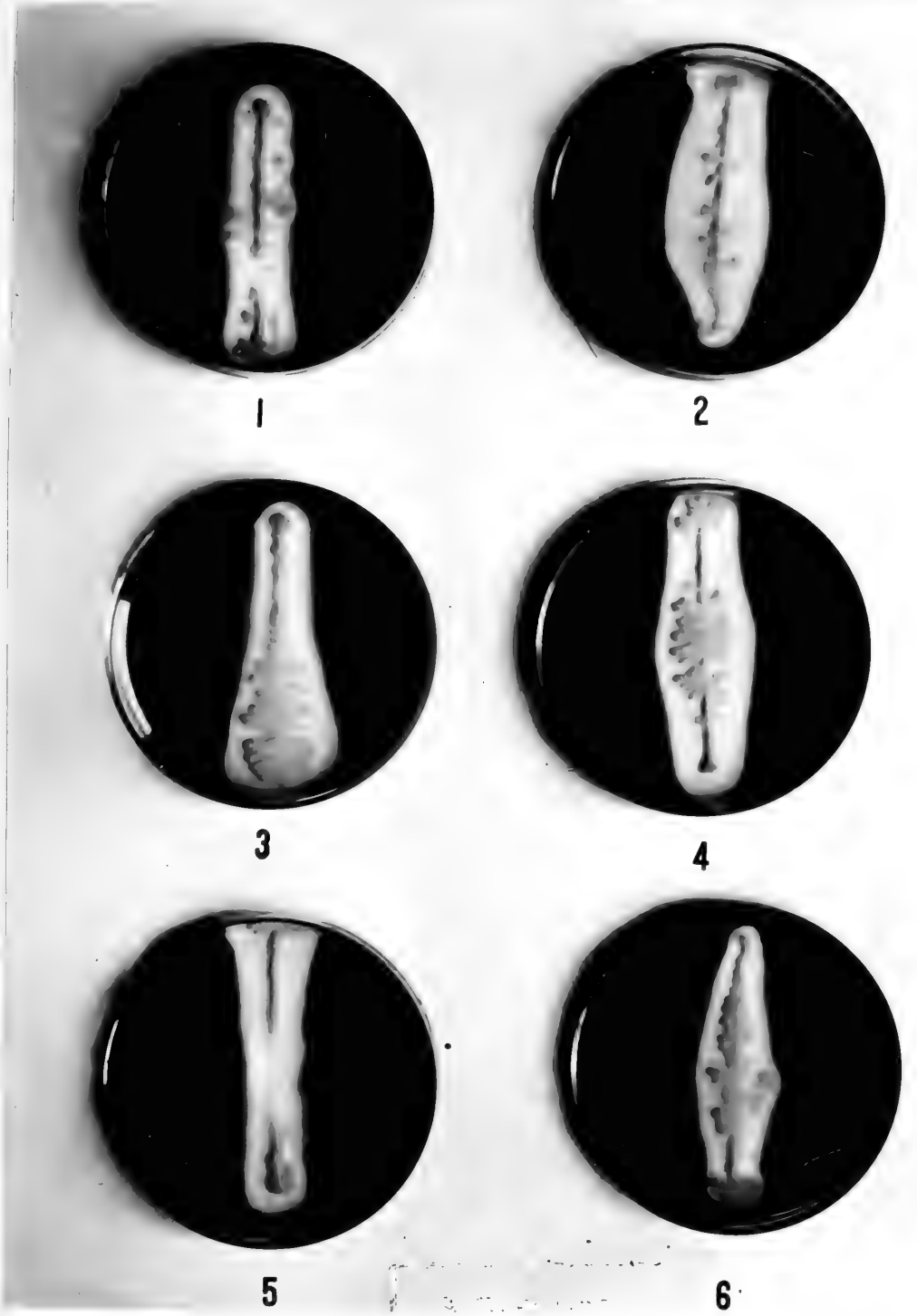


Fig. 4. Showing the Effect of Different Starches on Diastasic Action With B. vulgatus After Seven Days Incubation at 37°C on 2 1/2% Agar.

1 - Wheat Starch.
2 - Oat "
3 - Corn "
4 - Arrowroot Starch.
5 - Pea "
6 - Rice "

E. After determining by the plate method that 1% of soluble potato starch and 1 1/2% agar gave the best results for diastasic action, an attempt was made to determine the best method by the use of synthetic media using Frankel's Solution which has the following composition:

5 gms.	sodium chloride.
2 "	monocalcium phosphate.
6 c.c.	ammonium lactate.
4 gms.	asparagin.
1 liter	distilled water.
20 c.c.	n/1 sodium hydroxide.

Tubes of this medium containing 10 c.c. were prepared with increasing percentages of soluble potato starch from 1 to 5% inclusive. After 2, 3, 4, 5, 6 and 9 days, incubation at 37°C tests were made with Iodine in KI, and with Benedict's Solution, the results of which are given in Table V.

In the Iodine test, when necessary, both the Iodine and the starch media were diluted so as to detect the delicate shades of color which indicate the various stages in the decomposition of starch.

In the Benedict's test for reducing sugars, four c.c. of the starch solution to be tested were added to a tube containing five c.c. of Benedict's Solution. The mixture was then boiled over a bunsen flame for two minutes. Although the media with each percentage was brought to a boil before tubing, a perfect emulsion had not been formed, for after standing a short time even the 1% starch settled leaving a clear portion at the top of the tube. On longer incubation this clear portion increased, and in the tests made before stirring the contents of the tube, positive results were obtained sooner than in the thoroughly mixed portion.

Table Va. Diastasic Action in Frankel's Solution Tested with Iodine and Benedict's Solution.

Organisms.	Per cent of Starch	2 days				3 days				4 days				5 days				6 days			
		I		B		I		B		I		B		I		B		I		B	
		N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S	N	S
B. mesentericus	1	v	b	+	−	v	b	+	+	p	v	+	+	p	v	+	+	p	v	+	+
	2	"	"	+	−	"	"	+	+	v	"	+	+	"	"	+	+	"	"	+	+
	3	"	"	+	−			+	+	"	b	+	+	"	b	+	+	"	b	+	+
	4	"	"	+	−			+	+	"	"	+	+	"	"	+	+	"	"	+	+
	5			+	−			+	−			+	−	v		+	−	v		+	+
B. cereus.	1	b	b	−	−	b	b	−	−	b	b	−	−	b	b	−	−	b	b	−	−
	2	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−
	3	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−
	4	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−
	5	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−	"	"	−	−
B. vulgatus.	1	v	b	+	−	p	b	+	+	p	b	+	+	p	b	+	+	c	v	+	+
	2	"	"	+	−	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	3	"	"	+	−	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	4	"	"	+	−	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	5	p	"	+	−	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
B. arborescens.	1	p	b	+	+	p	b	+	+	p	b	+	+	c	v	+	+	c	v	+	+
	2	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	3	"	"	+	+	"	"	+	+	"	"	+	+	"	b	+	+	"	"	+	+
	4	v	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+	"	b	+	+
	5	"	"	+	−	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
B. subtilis.	1	v	b	+	+	p	b	+	+	v	b	+	+	c	b	+	+	c	v	+	+
	2	"	"	+	+	"	"	+	+	p	"	+	+	"	"	+	+	"	"	+	+
	3	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	4	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+	"	"	+	+
	5	"	"	+	−	"	"	+	+	c	"	+	+	"	"	+	+	"	b	+	+

I=Iodine.

B=Benedict's Solution.

N=Contents of tube not stirred.

S=Contents of tube stirred.

v=violet.
b=blue.
p=pink.
c=clear.

Table Vb. Diastasic Action in Frankel's Solution After 9 and 12 Days Incubation at 37°C.

Organism.	Percent of Starch.	9 days.				12days.			
		I		B		I		B	
		N	S	N	S	N	S	N	S
B. mesentericus.	1	c	v	+	+	c	p	+	+
	2	"	"	+	+	"	"	+	+
	3	"	b	+	+	"	v	+	+
	4	"	"	+	+	"	"	+	+
	5	"	"	+	+	"	b	+	+
B. cereus.	1	v	v	+	+	c	p	+	+
	2	"	"	+	+	"	"	+	+
	3	"	"	+	+	"	v	+	+
	4	"	b	+	+	"	"	+	+
	5	"	"	+	-	"	b	+	-
B. vulgatus.	1	c	v	+	+	c	c	+	+
	2	"	"	+	+	"	v	+	+
	3	"	"	+	+	"	"	+	+
	4	"	"	+	+	"	"	+	+
	5	"	"	+	+	"	"	+	+
B. arborescens.	1	c	p	+	+	c	c	+	+
	2	"	"	+	+	"	"	+	+
	3	"	v	+	+	"	p	+	+
	4	"	"	+	+	"	v	+	+
	5	"	"	+	+	"	"	+	+
B. subtilis.	1	c	p	+	+	c	c	+	+
	2	"	"	+	+	"	"	+	+
	3	"	v	+	+	"	p	+	+
	4	"	"	+	+	"	"	+	+
	5	"	"	+	+	"	v	+	+

I=Iodine.
B=Benedict's Solution.
N=Contents of tube not stirred.
S= " " " stirred.

c=clear.
p=pink.
v=violet.
b=blue.

This would indicate that the lighter top part was subject to diastasic action quicker than the heavier lower portion. This may also have been due to the fact that the test organisms were aerobic and grew more readily in the upper stretches of the medium.

As in previous results, B. cereus required a long incubation period for diastasic action to take place. B. arborescens and B. subtilis gave positive action on the second day with the smaller percentages of starch, and on the third day with all percentages. B. vulgatus gave no action with the stirred contents of the tube on the second day, but complete action on the third day. B. mesentericus did not give action until the fourth day, and B. cereus did not give action at all.

This shows variability in the different organisms as to the production of diastases.

Being unable to obtain asparagin, Uschinsky's Protein Free Medium (lacking the asparagin) was used, having the following composition:

Ammonium lactate	10.0gm.
Sodium chloride	5.0
Magnisium sulphate ($MgSO_4 \cdot 7H_2O$)	0.2
Calcium chloride ($CaCl_2$)	0.1
Dipotassium phosphate (K_2HPO_4)	1.0
Distilled water	1000 c.c.

To determine whether or not this media would sustain growth, each organism was inoculated into 5 c.c. of the medium and incubated at 37°C for two days; no growth occurred except in organism III where a scum formed. A pinch of ammonium chloride was added to tubes I and II, incubated and very slight growth occurred.

To determine the effect of the concentration of starch on the action of bacterial diastase, percentages of starch from 0.1 to 3.0 inclusive in the above media plus a pinch of ammonium chloride were prepared. The test organisms were inoculated into tubes containing 5 c.c. of this starch media and incubated at 37°C for five days. Controls were run with each per cent of starch. When tested on the fifth day with Iodine and Benedict, there was no evdence that action had taken place. Certain random tubes were tested in a hanging drop for the organism, and onplain agar streaks for growth. Growth resulted on all of the slants.

Since Uschinsky's media plus ammonium chloride and lacking asparagin did not give satisfactory results, plain broth was used with the same series of percentag es of starch, and tested after five days incubation at 37°C. The results in Table VI seem to indicate that 1% of starch is necessary to give the best results.

Table VI. Diastasic Action in Starch Broth After Five Days Incubation at 37°C.

Organisms.	Percentages of Starch.																							
	0.1		0.2		0.3		0.4		0.5		0.6		0.7		0.8		0.9		1.0		2.0		3.0	
	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B	I	B
B. Mesentericus.	fp	-	fp	-	fp	-	p	-	p	-	p	-	p	-	p	-	p	-	p	+	v	+	v	+
B. cereus.	p	-	p	-	v	-	v	-	bv	-	bv	-	bv	-	lv	-	bv	-	b	-	b	-	b	-
B. vulgatus.	fp	-	fp	-	fp	-	p	-	lv	-	lv	-	lv	-	v	-	v	-	v	+	v	+	v	+
B. arborescens.	c	-	c	-	c	-	c	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+	v	+
B. subtilis.	c	-	c	-	c	-	c	-	p	-	p	-	bv	+	bv	+	bv	+	bv	+	bv	+	bv	+

I=Iodine.
B=Benedict's Solution.

c=colorless
fp=faint pink.
p=pink.
lv=light violet.
v=violet.
bv=blue violet.
b=blue.

In order to determine whether or not a shorter incubation period would be a factor in the previous results (Table VI) percentages of starch 0.1, 0.2, 0.3 and 0.4 were prepared with plain Broth as before, incubated at 37°C and tested on 1, 2 and 3 days, respectively. The results as given in Table VII indicate that the time element may be a factor in the results obtained.

Table VII. Diastasic Action in Starch Broth with 0.1, 0.2, 0.3 and 0.4 percents of Starch, After Incubation at 37°C for 1, 2 and 3 Days.

Organisms.	One Day.							
	0.1%		0.2%		0.3%		0.4%	
	I	B	I	B	I	B	I	B
B. mesentericus.	fp	-	fp	-	lv	-	lv	-
B. cereus.	b	-	b	-	b	-	b	-
B. vulgatus.	v	-	v	-	b	-	b	-
B. arborescens.	p	-	lv	-	lv	-	lv	-
B. subtilis.	fp	-	v	-	v	-	v	-

Organisms.	Two Days.							
	0.1%		0.2%		0.3%		0.4%	
	I	B	I	B	I	B	I	B
B. mesentericus.	fp	-	fp	-	fp	-	fp	-
B. cereus.	b	-	b	-	b	-	b	-
B. vulgatus.	fp	-	fp	-	c	-	c	+
B. arborescens.	fp	-	p	-	lv	-	lv	+ -
B. subtilis.	fp	-	fp	-	lv	-	v	-

Organisms.	Three Days.							
	0.1%		0.2%		0.3%		0.4%	
	I	B	I	B	I	B	I	B
B. mesentericus.	c	-	c	-	fp	-	fp	-
B. cereus.	b	-	b	-	b	-	b	-
B. vulgatus.	c	-	c	-	c	-	c	+
B. arborescens.	c	-	p	-	lv	-	lv	+ -
B. subtilis.	c	-	c	-	p	-	v	-

I=Iodine.
B=Benedict's Solution.

fp=faint pink.
p=pink.
lv=light violet.
v=violet.
c=colorless.
b=blue

Having determined that the lower and higher percentages of starch in Plain Broth were not as satisfactory as 1% starch and that the time element is a factor, tests were made with different Hydrogen ion concentrations to determine whether or not reaction is important in the bacterial autolysis of starch.

In doing this, a series of tubes of different Hydrogen ion concentrations were prepared for each organism, using 1% potato starch in Plain Broth. The acidity was obtained by adding 2 drops of C. P. Acetic acid to 20 c.c. of distilled water and to each series of ten tubes (for each organism), each tube containing 5 c.c. of starch broth was added 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 drops. These tubes were sterilized, inoculated, incubated at 37°C and tested with Clark & Lubs Indicators; with Iodine and with Benedict's Solution on the third and fifth days. Uninoculated controls were incubated and tested with each of the series of different percents of starch.

Testing with Clark & Lubs was carried out by placing a drop of the starch broth to be tested into a depression in a test tablet, and adding a drop of the Indicator. The controls were tested also.

Evidently not enough acid had been added, for with even the 10 drops, Thymol blue, which should be very sensitive to acid, gave its alkaline color, yellow. Brom thymol blue with a P_H range of 6.0 - 7.6 also gave a positive test for alkalinity in the controls.

Most of the cultures gave an acid test with Brom thymol blue, Phenyl red, and Cresol red, in all of the tubes in the entire range of acidity used, while the other indicators gave tests for alkalinity in practically all of the tubes in the entire range of acidity used.

One per cent starch broth was prepared and added to flasks in amounts so that when the final addition was made with N/200 Hcl. and N/200 NaOH each of the ten flasks contained a total volume of 100 c.c.

Ten c.c. of the media were placed in each tube of the series of the ten different concentrations for each organism, sterilized, inoculated, and incubated at 37°C for five days. At the end of this time, the tubes were tested for Hydrogen ion concentration with the seven Clark & Lubs Indicators used in the preceding experiment, and with phenolphthalein. They were also tested for color change with Iodine, and for sugar with Benedict's Reagent.

The results which are given in Table VIII show that the correct range of acidity and alkalinity had not been used. The data on the Hydrogen ion as carried out here is not conclusive.

Table VIII. Diastasic Action in 1% Starch Broth with Different Amounts of Acidity and Alkalinity as Tested with Clark and Lubs Indicators, Iodine, Benedict's Solution, and Phenolphthalein.

Organisms.	Tubes.	Reaction.	Iodine.	Benedict.	Phenolphthalein.
B. mesentericus.	H.cl	5.2 - 6.8 6.8 - 7.6	lv	+	
	NaOH	4.4 - 6.0 6.0 - 7.6	"	+	acid.
B. cereus.	H.cl	4.4 - 6.0 6.0 - 7.6	blue	-	
	NaOH	4.4 - 6.0 6.0 - 7.6	"	-	acid.
B. vulgatus.	H.cl	all alkaline.	blue	+	
	NaOH	all alkaline.	"	+	faint alkaline.
B. arborescens.	H.cl	5.2 -6.8 6.0 - 7.6	lv	+	
	NaOH	5.2 -6.8 6.0 - 7.6	"	+	acid.
B. cereus.	H.cl	4.4 - 6.0 5.2 - 6.8	violet	-	
	NaOH	4.4 - 6.0 5.2 - 6.8	"	-	acid.
Control.	H.cl	all alkaline.		-	
	NaOH	all alkaline.		-	alkaline.

lv=light violet.

F. Edson and Carpenter's (1912) Method was then tried. Five c.c. of 1% starch paste containing 2% thymol was added to 5 c.c. of broth cultures of each of the test organisms and incubated over night at 37°C and tested with Iodine and Benedict's Solution.

Only one organism (B. vulgatus) gave positive results with Benedict's Solution. The same results were obtained on repeating the experiment.

Table IX. Diastasic Action According to Edson and Carpenter's Method, When Tested with Iodine and with Benedict's Solution.

Organisms.	Iodine.	Benedict.
B. mesentericus.	purple.	-
B. cereus.	light purple.	-
B. vulgatus.	clear.	++
B. arborescens.	light purple.	-
B. subtilis.	trace of pink.	+
Control.	purple.	-

Waksman's starch agar plates were prepared, streaked, incubated and tested as with former plates. This agar contains to a liter, 1gm. K_2HPO_4, 0.5 gm. M_gSO_4, 0.5 gm. Kcl., 0.01 gm. $FeSO_4$, 2 gm. $NaNO_3$, and 10 gm. of starch. On these plates the growth seldom exceeded 1 m.m. in diameter with any of the organisms. The plates were tested on 2, 5 and 7 days and the results given in Table Xa.

Waksman's tube method was used also. In this, the minerals and 1% starch boiled in distilled water, were diluted to 1 liter, tubed 10 c.c. to a tube, sterilized, inoculated, incubated, and tested with Iodine, and with Benedict's Solution for sugar. A control tube was used to check the height of starch in the tubes as an indication of liquefaction of the starch. As shown in Table Xb B. vulgatus was the only one that gave diastasic action as shown by the height of starch, by Iodine and by Benedict's Solution.

Table Xa. Diastasic Growth and Action in Millimeters with Waksman's Starch Plates.

Organisms.	2 days.		5 days.		7 days.		9 days.	
	Growth	Action	Growth	Action	Growth	Action	Growth	Action
B. mesentericus.	1	5(faint)	1	5(faint)	1	5(faint)	1	5(faint)
B. cereus.	1	-	1	-	1	-	1	-
B. vulgatus.	1	8	2	25	3	32	3	40
B. arborescens.	1	-	1	-	1	-	1	-
B. subtilis.	1	10(faint)	1	10(faint)	1	18(faint)	1	18(faint)

Table Xb. Diastasic Action with Waksman's Tube ethod when Tested with Iodine and with Benedict's Solution.

Organisms.	2 days			5 days			7 days			9 days		
	Y	I	B	Y	I	B	Y	I	B	Y	I	B
B. mesentericus.	1/3	blue	-	1/3	blue	-	1/3	blue	-	1/3	blue	-
B. cereus.	1/3	"	-	1/3	"	-	1/3	"	-	1/3	"	-
B. vulgatus.	1	pink	+	1	pink	+	1	pink	+	1	pink	+
B. arborescens.	1/3	blue	-	1/3	blue	-	1/3	blue	-	1/3	blue	-
B. subtilis.	1/3	"	-	1/3	"	-	1/3	"	-	1/3	"	-
Control.	1/3	"	-	1/3	"	-	1/3	"	-	1/3	"	-

Y=portion of tube in millimeters showing clearing by liquefaction.

I=Iodine.

B=Benedict's Solution.

Plates were then made according to Crabill and Reed's Method. This media contained the following formula:

1000	cc.	distilled water.
0.5	gm.	magnesium sulphate.
1.0	"	di-potassium hydrogen phosphate.
0.5	"	potassium chloride.
0.01	"	ferrous sulphate.
20.0	"	agar.

The starch in a suspension of cold water was added to the filtered agar, sterilized in flasks, and poured with frequent shaking into steril petri dishes, hardened and streaked as the previous plates were. These were incubated at 37°C for two, six and ten days, then flooded with Iodine solution and the results given in Table XI, As with Waksman's Method, B. vulgatus was the only organism that gave action.

Table XI. Growth and Diastasic Action in Millimeters According to Crabill and Reed's Plate Method.

Organisms.	Two Days		Six Days		Ten Days	
	Growth	Action	Growth	Action	Growth	Action
B. mesentericus.	1	-	1	-	1	-
B. cereus.	1	-	1	-	1	-
B. vulgatus.	1	5	1	12	1	25
B. arborescens.	1	-	1	-	1	-
B. subtilis.	1	-	1	5(faint)	1	5(faint)

An attempt was made to determine the efficiency of the spot inoculation in the center of the starch agar plate as compared to the streak method. In this, 1 1/2% starch agar was poured into sterile petri dishes, hardened and a spot of inoculation placed on the center of the agar. These plates were inverted, incubated at 37°C and tested with Iodine after one, two, five and seven days with results as given in Table XII. The growths resulting were almost perfect circles.

Table XII. Growth and Action in Millimeters on 1 1/2% Agar from Inoculation in the Center of the Plate.

Organisms.	1 day		2 days		5 days		7 days	
	Growth	Action	Growth	Action	Growth	Action	Growth	Action
B. mesentericus	6.	13	25	27	48	61	54	64
B. cereus.	7.	8	20	25	41	47	60	68
B. vulgatus.	8.	12	10	25	42	50	49	61
B. arborescens.	5.5	8.5	55	60	+	+	+	+
B. subtilis.	10	8.5	25	35	35	51	70	82

Since peptone is a food for growth, an attempt was made to determine the effect of varying the amount in the starch agar. Starch agar plates containing 1% starch were made with varying amounts of peptone as follows: 0.2%, 0.4%, 0.6%, 0.8% and 1.0%. These starch plates were prepared as the former ones were, and inoculation made by both the dot and streak method. After incubation for 2, 5, and 7 days, they were flooded with Iodine and the results in tables. The dot method gave an almost perfect circle of growth and diastasic action.

Table 13. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 1.0% Peptone and 1.0% Starch, and 1 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	10	28	10	28	20	47	12	51	41	66	25	50
B. cereus	14	22	9	19	30	43	15	35	33	41	25	61
B. vulgatus	20	26	10	22	45	57	26	56	20	56	10	56
B. arborescens	10	30	9	30	29	56	17	51	30	63	30	65
B. subtilis	21	34	16	34	37	75	39	65	60	75	50	78

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 14. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.8% Peptone, 1.0% Starch and 1 1/2% Agar.

Organism.	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	11	28	10	25	30	47	22	45	60	68	45	55
B. cereus	14	20	11	17	35	45	25	40	42	55	30	43
B. vulgatus	18	28	6	24	38	53	17	45	44	71	40	60
B. arborescens	4	27	3	28	20	50	16	47	52	57	60	63
B. subtilis	25	37	21	36	37	62	30	57	62	76	57	74

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 15. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.6% Peptone, 1.0% Starch and 1 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	10	20	2	21	23	30	45	54	50	55	63	70
B. cereus	11	18	11	18	21	34	18	32	28	42	25	41
B. vulgatus	15	30	9	21	10	40	22	33	19	54	10	46
B. Arborescens	10	25	2	26	53	62	58	75	75	90	++	++
B. subtilis	17	36	15	38	25	50	27	47	39	61	45	58

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 16. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.4% Peptone, 1.0% Starch and 1 1/2% Agar.

Organism	2 Days.				5 Days.				7 Days.			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	32	40	28	35	41	50	45	52	Ent	Ent	Ent	Ent
B. cereus	10	20	9	19	16	26	11	25	30	45	23	40
B. vulgatus	8	20	4	21	6	28	7	28	20	45	7	44
B. arborescens	5	25	37	45	55	65	55	64	68	75	75	80
B. subtilis	15	32	15	33	20	40	20	41	44	70	36	67

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 17. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.2% Peptone, 1.0% Starch and 1 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	32	38	37	43	-	-	70	80	++	++	70	84
B. cereus	10	20	7	19	21	82	20	28	35	45	25	37
B. vulgatus	3	16	2	21	13	37	9	37	15	50	10	50
B. arborescens	50	60	50	55	70	++	60	80	65	80	70	85
B. subtilis	11	32	10	30	37	56	25	55	30	63	30	65

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 18. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 1.0% Peptone, 1.0% Starch and 2 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	33	36	24	31	55	75	42	62	58	80	45	70
B. cereus	15	23	7	20	27	38	20	34	35	47	27	42
B. vulgatus	14	25	7	22	23	50	18	45	25	51	27	52
B. arborescens	32	42	27	37	58	72	45	63	53	82	48	78
B. subtilis	8	24	10	29	40	58	35	58	52	70	45	60

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 19. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Contaning 0.8% Peptone, 1.0% Starch and a 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streake		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	13	26	14	25	65	72	57	67	78	85	62	70
B. cereus	15	20	10	16	25	36	19	28	36	43	25	43
B. vulgatus	9	20	5	19	16	40	7	39	23	50	20	45
B. arborescens	20	28	22	27	60	68	44	54	64	72	55	68
B. subtilis	14	30	13	30	28	56	25	55	35	65	33	65

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 20. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.6% Peptone, 1.0% Starch and 2 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	27	34	21	32	60	66	53	63	70	75	80	90
B. cereus	13	20	9	20	24	32	20	29	29	38	23	43
B. vulgatus	11	25	2	20	19	40	8	35	23	70	16	43
B. arborescens	19	31	15	30	57	65	40	55	70	81	50	63
B. subtilis	18	32	11	35	30	58	25	55	38	66	87	63

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 21. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.4% Peptone, 1.0% Starch and 2 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	AA	Gr.	A
B. mesentericus	35	40	24	30	67	81	65	75	72	80	70	80
B. cereus	9	19	6	17	24	38	13	32	29	40	21	40
B. vulgatus	7	23	5	18	25	45	10	39	22	40	12	45
B. arborescens	34	42	28	38	65	85	47	67	73	87	50	75
B. subtilis	11	30	8	30	34	60	18	50	37	62	25	60

Gr. = Width of Growth in Millimeters.
A = Diastasic Action.

Table 22. Growth and Diastasic Action in Millimeters on Potato Starch Agar Plates Containing 0.2% Peptone, 1.0% Starch and 2 1/2% Agar.

Organism	2 Days				5 Days				7 Days			
	Dot		Streak		Dot		Streak		Dot		Streak	
	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A	Gr.	A
B. mesentericus	17	28	12	25	30	47	22	40	48	59	30	46
B. cereus	9	16	6	16	20	28	13	22	22	33	22	32
B. vulgatus	7	22	3	20	11	30	9	30	13	43	10	41
B. arborescens	10	26	7	25	14	40	8	37	27	49	27	49
B. subtilis	10	31	7	30	15	43	15	44	17	48	20	48

Gr = Width of Growth in Millimeters.
A = Diastasic Action.

Discussion.

In considering the results obtained in these experiments on the use of various methods which have been proposed in the past for determining diastasic action of microörganisms, it was found that 1% of potato starch after incubation at 37°C gave the best results. Smaller percentages showed a rather complete utilization of starch. A heavier agar (2 1/2%) prevented the diffusion of the enzyme and consequently gave a more concentrated action within a limited area.

As to the method of inoculation of starch plates both the dot and streak methods were used. Several points may be given in favor of the former method: ease and quickness of inoculation, ease of getting the radius of action and the better applicability to the group number.

In synthetic media different results were obtained. With Frankel's solution as a basis, one organism (B. cereus) which on the ordinary starch plate gave strong diastasic action gave no action except after long incubation. Uschinsky's Protein Free Medium as a basis was not satisfactory with percents of starch lower than 1%, and even then this same organism (B.cereus) remained negative as to diastasic action.

With Edson and Carpenter's tube method the 2% thymol evidently killed the enzymes formed as well as the organism with all except B. vulgatus and B. subtilis as these were the only ones which gave positive indication of diastasic action.

With Waksman's starch plates the width of growth of the organisms was greatly reduced and here only one organism (B.vulgatus)

gave positive diastasic action. The plate and tube method checked as to the action taking place.

The results with Crabill and Reed's method in which starch is the only source of carbon and in which there is no protein material for food for the organisms, the results showed that B. vulgatus was the only organism which gave positive action.

The whole question seems to be the point of getting an equilibrium between the amount of starch, the amount of enzyme, and the time. The main variations in the experiments were starch and time and enzyme, the variation in agar and peptone being practically a variation in enzyme.

Conclusion.

With lower percentages of starch (.010, .015, .020 and .025%) in 1 1/2% agar the amount of starch present did not give satisfactory results--the diastasic action with Iodine being hard to distinguish. The best results were obtained between 0.5% and 2.0% of starch showing that this amount probably furnished the proper concentration of starch to be attacked by the enzyme.

The amount of agar used has an effect upon the diffusibility and consequent action of the diastasic enzyme. With heavier agar (2 1/2%) the growth was not as extensive as with 1 1/2% but the diastasic action was more distinct showing that the heavier agar probably prevented the diffusion of the enzyme thus causing the action to be confined within narrower limits. One per cent of starch seemed to give the best results.

The length of incubation period is an important factor in the diastasic action of enzymes. The amount of starch used seems to depend upon the period of incubation--lower percents being better for the shorter period.

Different starches have an effect in the action of diastasic enzymes on the starch agar plates. The starches studied range in action from strongest to the weakest as follows: potato starch, arrowroot, pea, corn, wheat, oat, and rice.

A study of diastasic action in synthetic media showed variability in the different organisms as to the production of diastase.

With starch broth, one per cent of starch gave the best results. The time element, however, is probably a factor.

Waksman's plate and tube method, and Crabill and Reed's plate method, as tested with the five organisms, gave positive diastasic action with only one organism. (B. vulgatus). Edson and Carpenter's tube method gave positive action with two organisms (B. vulgatus and B. subtilis).

Variation in the amount of peptone used apparently had no influence on the amount of growth.

Acknowledgment.

All of the work reported in this paper was done while the author held an assistantship in the Bacteriology Department of the University of Illinois. The study was outlined by Dr. F. W. Tanner and it is a pleasure to acknowledge his advice and assistance given throughout the work.

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